PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [4] :		(11) International Publication Number:	WO 88/ 04319
C12N 1/20, 1/00	A1	(43) International Publication Date:	16 June 1988 (16.06.88)

(21) International Application Number: PCT/US87/03290

(22) International Filing Date: 4 December 1987 (04.12.87)

(31) Priority Application Number: 938,190

(32) Priority Date: 5 December 1986 (05.12.86)

(33) Priority Country: US

(71) Applicant: OXYRASE, INC. [US/US]; 298 N. Countryside Drive, Ashland, OH 44805 (US).

(72) Inventors: ADLER, Howard, I. ; 128 Indian Lane, Oak Ridge, TN 37830 (US). COPELAND, James, C. ; 298 N. Countryside Drive, Ashland, OH 44805 (US).

(74) Agent: FLOYD, John, P.; 704 N. Armistead St., Alexandria, VA 22312 (US).

(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BG, BJ (OAPI patent), BR, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CM (OAPI patent), DE, DE (European patent), DK, FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: MATERIAL AND METHOD FOR PROMOTING GROWTH OF ANAEROBIC BACTERIA

(57) Abstract

A material and method for promoting the growth of anaerobic bacteria which includes a nutrient media containing a hydrogen donor and sterile membrane fragments of mitochondria having an electron transfer system which reduces oxygen to water. Dissolved oxygen in the medium is removed by adding the sterile membrane fragments to the nutrient medium and holding the medium at a temperature of about 10°C to about 60°C until the dissolved oxygen is removed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**FR**	France	**ML**	Mali
AU	Australia	**GA**	Gabon	**MR**	Mauritania
BB	Barbados	**GB**	United Kingdom	**MW**	Malawi
BE	Belgium	**HU**	Hungary	**NL**	Netherlands
BG	Bulgaria	**IT**	Italy	**NO**	Norway
BJ	Benin	**JP**	Japan	**RO**	Romania
BR	Brazil	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SN**	Senegal
CH	Switzerland	**LI**	Liechtenstein	**SU**	Soviet Union
CM	Cameroon	**LK**	Sri Lanka	**TD**	Chad
DE	Germany, Federal Republic of	**LU**	Luxembourg	**TG**	Togo
DK	Denmark	**MC**	Monaco	**US**	United States of America
FI	Finland	**MG**	Madagascar		

MATERIAL AND METHOD FOR PROMOTING GROWTH OF ANAEROBIC BACTERIA

Field of the Invention

The present invention relates generally to promoting the growth of anaerobic bacteria and in particular relates to the use of sterilized mitochondrial derivatives for the production of anaerobic conditions in media to promote the growth of anaerobes.

Many of the bacteria present in natural environments are sensitive to oxygen and will not grow in its presence. When these organisms (known as anaerobes) are brought into the laboratory, it is often necessary to employ cumbersome physical and chemical techniques in order to get them to grow. Some of these bacteria produce diseases of man and related species. Others produce important industrial end products such as methane, hydrogen and various alcohols. The manipulation of these organisms is, to some degree, limited by the ease with which they can be grown.

In order to grow anaerobes, it is necessary for the medium in which the anaerobe is to be grown to be substantially free of oxygen. Oxygen can be removed fairly efficiently from such media by sparging with high purity nitrogen or other inert gas. However, these liquid media are subject to foaming so that this process presents a number of mechanical difficulties. Further, after sparging is stopped, the medium is easily recontaminated with oxygen.

Oxygen can also be removed from liquid media by the addition of reducing agents. However, most of these agents are strong reducing agents and any residual agent or its by-products in the media tends to inhibit the subsequent growth of anaerobes in the media. Also, the reducing agent which is consumed during the initial removal of oxygen is not available to act upon oxygen which might later find its way into the system.

In the case of a solid medium, such as agar, oxygen

removal with an inert gas is difficult to accomplish because the oxygen in the medium comes to equilibrium with the inert atmosphere slowly and never reaches a zero concentration. Reducing agents can be added to a solid media but again there is still the problem of the inhibition of anaerobic growth in the media due to any residual reducing agent or its by-products as well as the problem of providing the system with the ability to consume any oxygen which might later find its way into the system.

United States Patent 4,476,224, issued October 9, 1984, to Howard I. Adler, discloses that sterilized membrane fragments from certain oxygen-consuming bacteria may be incorporated into media to remove oxygen rapidly and completely from the media. This patent teaches that use of these membrane fragments overcomes many of the problems recited above.

The principle object of this invention is to provide a material with similar oxygen-consuming properties, but derived from a distinctly different, non-bacterial source. More specifically, this invention provides oxygen consuming enzyme systems from mitochondria obtained from a variety of higher non-bacterial organisms, as well as a method for using such systems to grow anaerobic bacteria. The oxygen-consuming enzyme systems of this invention, obtained from mitochondria exhibit properties which make them useful for increasing the range of anaerobic bacteria that can be grown using the membrane fragments obtained from bacteria disclosed in U.S. 4,476,224. Other objects and advantages of the invention will become apparent from the following descriptions of various embodiments and examples of the invention.

In accordance with this invention, when membrane fragments from oxygen-consuming mitochondria are

incorporated into media, either liquid or solid, oxygen is rapidly and completely removed from the media. As will be pointed out, in some instances it is necessary to incorporate in the media a small amount of a suitable hydrogen donor so that reduction of the oxygen to water is facilitated. The sterile membrane fragments of this invention can be employed without foaming problems. They are non-toxic and the presence of these membranes has no adverse effect upon the growth of the anaerobic bacteria in the media, even when used at high levels, such as a ten-fold excess. Further the presence of the membrane fragments in the media provides the media with the capacity to reduce additional oxygen which later may enter the system so that extreme methods of sealing the system are not required.

A great number of fungi, yeasts, and plants and animals have mitochondria that reduces oxygen to water, if a suitable hydrogen donor is present in the medium. Some of the sources of oxygen reducing membranes from these mitochondria are: beef heart muscle, potato tubers, spinach, Saccharomyces, Neurospora, Aspergillus, Euglena and Chlamydomonas. The process of producing the useful membrane fragments involves the following steps:

1. Yeast, fungal cells, algae and protozoa, having mitochondria membranes containing an electron transfer system which reduces oxygen to water can be isolated, are grown under suitable conditions of active aeration and a temperature which is conducive to the growth of the cells, usually about 20°C to 45°C in a broth media. Alternately, mitochondria may be obtained from cells of animal or plant origin.

2. The cells are collected by centrifugation or filtration, and are washed with distilled water.

3. For the preparation of crude mitochondrial membrane fragments a concentrated suspension of the cells is treated to break up the cell walls and mitochondria. This is accomplished by known means, for example by ultrasonic treatment or by passing the suspension several times through a French pressure cell at 20,000 psi.

4. The cellular debris is removed by low speed centrifugation or by microfiltration (cross-flow filtration).

5. The supernatant or filtrate is subjected to high speed centrifugation (175,000Xg at 5°C) or ultrafiltration.

6. For the preparation of material of higher purity, the cells of step 2 are suspended in a buffer containing 1.0M sucrose and are treated by means which break up the cell walls or membranes but leave the mitochondria intact. This is accomplished by known means, for example, by ultrasonic treatment, passage through a French pressure cell at low pressure, enzymatic digestion or high speed blending with glass beads.

7. The cellular debris from step 6 is removed by differential centrifugation or filtration.

8. The supernatant or retentate from step 7 is passed through a French Press at 20,000 psi to break the mitochondria into small pieces.

9. Mitochondrial debris from step 7 is removed by centrifugation at 12,000Xg for approximately 15 minutes or by microfiltration.

10. The supernatant or filtrate from step 9 is subjected to high speed centrifugation (175,000Xg at 5°C) or ultrafiltration.

11. The pellet or retentate from step 5 (crude mitochondrial fragments) or the pellet or retentate

from step 10 (purified mitochondrial membrane fragments) are resuspended in a buffer solution at a pH of about 7.0 to about 7.5. A preferred buffer solution is 0.02M solution of N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES).

12. The membrane fragments in the buffer solution are then passed under pressure through a filter having openings of a size which will retain any intact microorganisms to effect sterilization. Openings of about 0.2 microns are satisfactory.

13. The sterilized suspension is then preferably used promptly or stored for use at about -20°C or it may be freeze dried.

In use, a small amount of the sterile membrane fragment suspension is added to a liquid medium which is to be used for the growth of the anaerobic bacteria (about 25 to 3000 mg of fragments per liter of medium). The medium is permitted to stand for a short period of time at a temperature of from about 10°C to about 60°C until the oxygen is consumed. This action takes up to about 20 to 30 minutes, depending upon the concentration of the sterile membrane fragments and the temperature. At concentrations of about 500 mg/l and temperatures of about 35°C, removal is effected in from about 2-8 minutes. After the oxygen is removed, an inoculum of anaerobic bacteria is introduced into the medium. The inoculated medium is then incubated for the growth period at the proper temperature for the bacteria which are to be grown. Preferably, the air space above the liquid medium in its container is flooded with an inert gas such as nitrogen. This minimizes the amount of oxygen that must be removed by the membrane system and prolongs the life of the oxygen-consuming system. This also gives assurance that, if there is an accidental leak of air into the system, the system will consume that air

and insure that the growth of the anaerobic bacteria will not be retarded.

In the case of a solid medium, such as agar, the membrane preparation is preferably added to the medium in a molten state at approximately 45°C at a level of about 25 to 3000 mg of fragments per liter of medium. The medium is then poured into Petri dishes, or the like, in which it is held at a temperature of from about 10°C to about 60°C until the oxygen is consumed, usually in a period of time less than 20 to 30 minutes. The time of removal depends upon the temperature and concentration of the sterile membrane fragments, as pointed out above. The medium is then inoculated with the anaerobe to be grown and incubated at the proper temperature for growth. Again, the Petri dishes should preferably be maintained in an atmosphere of inert gas, such as nitrogen, but good results can be obtained on rapidly growing anaerobes without such a precaution since the membrane system is capable of consuming reasonable amounts of oxygen from any air which may leak into the dish.

In the event that a synthetic media is employed, it may be necessary to add a small amount of a hydrogen donor which does not interfere with the growth of the selected anaerobic bacteria. Suitable hydrogen donors are sodium lactate, sodium succinate, alpha-glycerol phosphate, alpha-keto gluterate or sodium formate. Most natural media do not require the addition of a hydrogen donor, but with some media, particularly synthetic media, the addition of the hydrogen donor is necessary for the sterile membrane fragments to perform their oxygen removing function.

Some 18 species of anaerobic bacteria representing 8 different genera will flourish in media which has had its oxygen removed by the sterile membrane system of this invention. Because of the fact that the sterile membrane fragments are in the form of particles which do not

penetrate the cell walls the system does not adversely affect the anaerobes being grown. This is in contrast to a system in which a chemical reducing agent is used to accomplish the removal of dissolved oxygen when the residual reducing agent or its by-product may penetrate the cell walls. Thus, because the anaerobic bacteria are being grown under more natural conditions, they flourish at a greater rate than in media which has been treated with such reducing agents.

EXAMPLE I

A nutrient broth is inoculated with Saccharomyces cervisiae (ATCC 18790). The nutrient broth employed is Malt Extract Broth sold by Difco Laboratories, Detroit, Michigan. The inoculated broth is maintained at 24°C and is actively aerated. The growth is continued until it is in the late logarithmic phase.

The broth containing the Saccharomyces cervisiae cells is centrifuged at 4,000Xg to harvest the yeast cells. The cells are washed with distilled water and centrifuged. This washing and centrifuging are repeated.

The harvested cells are cooled to 0-2 °C and suspended in two volumes of a buffer containing 1.0M sucrose, 0.02M tris-(hydroxymethyl)amino methane (Tris) and 0.0001 sodium ethylene diamine tetra acetate (EDTA) at pH 7.4. To this suspension is added an equal volume of glass beads (average diameter 0.15mm) and the mixture is homogenized at 15,000 RPM in a steel blender for 3-4 minutes. The glass beads are allowed to settle and the supernatant is decanted.

The supernatant is centrifuged at 2400Xg for 20 minutes to remove unbroken cells and debris. The mitochondria are harvested from the supernatant by centrifuging at 20,000Xg for 15 minutes.

The mitochondria are then resuspended in 0.02M HEPES buffer at pH 7.5 (CalBiochem-Behring Corporation, LaJolla, California) and passed three times through a French pressure cell at 20,000 psi.

The suspension of the membrane fragments in the buffer solution is then passed through a 0.22 micron filter under pressure to produce the sterile membrane fragments to be used to produce anaerobiosis in the media to be used for growing anaerobes. The suspension of membrane fragments is stored at about -20°C. The dry weight of the solids in the suspension is about 30 mg/ml which is determined by desiccating samples over phosphorous pentoxide in a vacuum.

EXAMPLE II

Ten microliters of the suspension of sterilized membrane fragments from Example I is added per milliliter of oxygen saturated Difco Nutrient Broth at 37°C. In five minutes all of the oxygen is removed. A portion of the nutrient broth, treated with the sterilized membrane fragments and held until anaerobic conditions are obtained, is inoculated with Clostridium difficile and incubated at 37°C for 16 hours in a sealed container. A luxuriant growth (approximately 10/9 cells per ml) is observed.

EXAMPLE III

Ten microliters of the sterile membrane fragment preparation of Example I is added per milliliter of synthetic medium which is supplemented by the addition of sodium lactate to a final concentration of 0.25M. The synthetic medium consists generally of inorganic salts, ethanol and sodium acetate. It is prepared from reagent grade chemicals. A sample of medium containing the membrane fragments is held at 37°C to remove oxygen and

is inoculated with Clostridium kluyveri. The inoculated medium is incubated at 34°C for 96 hours. At the end of that time a luxuriant growth (approximately 10/9 cells per ml) is observed.

EXAMPLE IV

The sterile membrane fragment suspension of Example I is added to molten agar at 45°C at a level of 10 microliters of suspension per milliliter of agar. The agar is poured into a Petri dish and held to obtain anaerobic conditions. It is inoculated with Clostridium kluyveri and incubated in the presence of an inert gas atmosphere at a temperature of 34°C for a period of 96 hours. Colonies with a diameter of approximately 2 millimeters are observed at that time.

Similar results are observed with sterilized membrane fragments from beef heart muscle, potato tubers, spinach, Saccharomyces, Neurospora, Aspergillus, Euglena and Chlamydomonas. In all cases the concentration of membrane fragments in the sterile membrane suspension is about 25 - 30 mg/ml. At 37°C all of the oxygen is removed from the nutrient media in from about 2 to about 8 minutes when the membrane fragment suspension is present at from about 10 to about 100 milliliters per liter of broth (250-3000 mg of fragments per liter of broth). At higher levels, oxygen removal is more rapid than at lower levels.

As pointed out above, when a synthetic media or one not containing a hydrogen donor is employed, a hydrogen donor which is compatible with the bacteria being cultured should be employed to supplement the medium. The hydrogen donor, e.g. sodium lactate, sodium succinate, alpha-glycerol phosphate, or sodium formate should be employed at a level of the order of 0.15 to 0.25M.

Anaerobic bacteria which can be successfully grown in nutrient media treated by the sterile membrane system described herein are:

Clostridium difficile
Clostridium tetani
Clostridium kluyveri
Clostridium sporogenes
Clostridium perfringens
Clostridium sordelli
Clostridium butyricum
Clostridium bifermentans
Clostridium acetobutyricum
Peptostreptococcus anaerobius
Peptostreptococcus micros
Peptostreptococcus magnus
Desulfovibrio vulgaris
Fusobacterium nucleatum
Viellonelia parvula
Propionibacterium acnes
Eubacterium limosum
Bacteriodes fragilis

The use of sterile membrane-containing media may be used in clinical laboratories to stimulate growth of anaerobic bacteria from human patients. Sterile membrane containing media may be used to increase the survival of anaerobes in medium used to transport samples from the patient to the laboratory and also for the determination of antibiotic sensitivity patterns of anaerobic bacteria. It also has use in producing the anaerobic conditions required in many industrial fermentation processes. The use of sterile membrane fragments, as described above, produce little or no toxic side effects when used in amounts much greater than those required to achieve oxygen-free

conditions. Small quantities of the membrane fragments reduce a medium that is initially saturated with oxygen to an anaerobic condition and maintain that condition even though small amounts of air are introduced.

Various features of the invention are set forth in the appended claims.

What is claimed is:

1. A nutrient medium for growing anaerobic bacteria which includes a hydrogen donor and sterile membrane fragments derived from mitochondria having membranes containing an electron transfer system which reduces oxygen to water.

2. The nutrient medium of claim 1 in which the sterile membrane fragments are present in a concentration of from about 25 to 3000 mg/l.

3. A nutrient medium for growing anaerobic bacteria which includes a hydrogen donor and sterile membrane fragments derived from yeast, fungi, plants, and animals selected from the class consisting of beef heart muscle, potato tubers, spinach, Saccharomyces, Neurospora, Aspergillus, Euglena and Chlamydomonas.

4. The nutrient medium of claim 3 wherein the sterile membrane fragments are derived from yeast and fungi selected from the class consisting of beef heart muscle, potato tubers, spinach, Saccharomyces cervisiae, and Aspergillus oryzae.

5. The nutrient medium of claim 1 wherein the hydrogen donor is selected from the group consisting of sodium lactate, sodium succinate, alpha-ketoglutarate, sodium formate and alpha-glycerol phosphate.

6. The method of removing dissolved oxygen from a nutrient medium for anaerobic bacteria comprising the steps of introducing sterile membrane fragments derived from mitochondrial membranes which contain an electron transfer system which reduces oxygen to water in the presence of a hydrogen donor to the nutrient medium and maintaining the medium containing sterile membrane fragments at a temperature of from about 10°C to about 60°C until the dissolved oxygen is converted to water.

7. The method of claim 6 further comprising the step of adding a hydrogen donor to the nutrient medium.

8. The method of claim 7 wherein the hydrogen donor is selected form the group consisting of sodium lactate, sodium succinate, alpha-ketoglutarate, sodium formate, and alpha-glycerol phosphate.

9. A method of removing dissolved oxygen from a nutrient medium for anaerobic bacteria comprising the steps of introducing sterile membrane fragments, derived from yeasts, fungi, plants and animals selected form the class consisting of beef heart muscle, potato tubers, spinach, Saccharomyces, Neurospora, Aspergillus, Euglena and Chlamydomonas, to the medium and maintaining the medium containing sterile membrane fragments at a temperature of from about 10°C to about 60°C until the dissolved oxygen is converted to water.

10. The method of claim 9 wherein the sterile membrane fragments are derived from yeast and fungi selected from the class consisting of beef heart muscle, potato tubers, spinach, Saccharomyces cervisiae, and Aspergillus oryzae.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/03290

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12N 1/20, 1/00

U.S.Cl: 435/253,262

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/253,262

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category * [15]	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	US, A, 4,476,224 Published 09 October 1984. (ADLER), note Abstract.	1-10
Y	Journal of Bacteriology, Volume 147, No. 2, issued August 1981 (Williams and Wilkins, Co., Baltimore, Maryland USA). (ADLER ET AL) "Cytoplasmic Membrane Fraction That Promotes Septation in an Escherichia coli Ion Mutant", pages 326-332, See particularly pages 326 and 329.	1-10
Y	Biotechnology and Bioengineering Symposium No. 11, issued May 1981 (John Wiley & Sons, Inc. New York, USA). (ADLER ET AL) "A Novel Approach to the Growth of Anaerobic Microorganisms", pages 533-540, See particularly Summary (page 533) and page 534 and 539.	1-10

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
11 FEBRUARY 1988	

International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	Lionel Shapiro

Form PCT/ISA/210 (second sheet) (May 1986)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Biochemistry issued 1979 (Academic Press, Inc., New York, USA) (METZLER) See page 571.	1-10

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.